

Exhibit A: Listing of Claims

Appln No. 09/892,591

January 6, 2005 Amendment and Reply to

Office Action dated October 6, 2004

Claims 1-34 (canceled)

35. (currently amended) A method for producing a recombinant glycoprotein in a lower eukaryotic host cell that does not display an alpha-1,6 mannosyltransferase activity with respect to the N-glycan on a glycoprotein, the method comprising the step of introducing into the ER or Golgi apparatus of the host cell a mannosidase enzyme comprising:

(a) a catalytic domain having alpha-1,2 mannosidase activity selected to have a pH optimum within 1.4 pH units of the average pH optimum of glycosylation-related enzymes in the subcellular location where the domain is targeted; and

(b) a cellular targeting signal peptide not normally associated with the catalytic domain selected to target the catalytic domain to said ER or Golgi apparatus;

whereby, upon expression of the recombinant glycoprotein in the host cell, in excess of 30 mole % of the N-glycan structures attached thereto have a $\text{Man}_5\text{GlcNAc}_2$ glycoform that can serve as a substrate for GlcNAc transferase I *in vivo*.

36. (canceled)

37. (canceled)

38. (canceled)

39. (previously amended) The method of claim 35, wherein the mannosidase is targeted to the early, medial, late Golgi or the trans Golgi network of the host cell.

40. (previously amended) The method of claim 35, further comprising the step of introducing into the host cell one or more additional enzymes selected from the group consisting of mannosidases, glycosyltransferases and glycosidases.

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41. (canceled)
42. (canceled)
43. (canceled)
44. (previously amended) The method of claim 35 or 40, wherein the glycoprotein comprises one or more sugars selected from the group consisting of N-acetylglucosamine, galactose, sialic acid, and fucose.
45. (previously amended) The method of claim 35 or 40, wherein the glycoprotein comprises at least one oligosaccharide branch comprising the structure NeuNAc-Gal-GlcNAc-Man.
46. (previously amended) The method of claim 35 or 40, wherein the host is selected from the group consisting of *Pichia pastoris*, *Pichia finlandica*, *Pichia trehalophila*, *Pichia koclamae*, *Pichia membranaefaciens*, *Pichia opuntiae*, *Pichia thermotolerans*, *Pichia salictaria*, *Pichia guercuum*, *Pichia pijperi*, *Pichia stiptis*, *Pichia methanolica*, *Pichia* sp., *Saccharomyces cerevisiae*, *Saccharomyces* sp., *Hansenula polymorpha*, *Kluyveromyces* sp., *Candida albicans*, *Aspergillus nidulans*, and *Trichoderma reesei*.
47. (previously amended) The method of claim 35 or 40, wherein the host is deficient in the activity of one or more enzymes selected from the group consisting of mannosyltransferases and phosphomannosyltransferases.
48. (previously presented) The method of claim 47, wherein the host does not express an enzyme activity with respect to the N-glycan on a glycoprotein, the activity selected from the group consisting of 1,6 mannosyltransferase; 1,3 mannosyltransferase; and 1,2 mannosyltransferase.

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49. (previously amended) The method of claim 35 or 40, wherein the host is an OCH1 mutant of *P. pastoris*.

50. (previously amended) The method of claim 35 or 40, wherein the host expresses one or more enzymes selected from: GnTI; a UDP-specific diphosphatase; a GDP-specific diphosphatase; and a UDP-GlcNac transporter.

51. (canceled)

52. (previously amended) The method of claim 35 or 40, further comprising the step of isolating the glycoprotein from the host.

53. (previously presented) The method of claim 52, further comprising the step of subjecting the isolated glycoprotein to at least one further glycosylation reaction *in vitro*, subsequent to its isolation from the host.

54. (previously amended) The method of claim 35, further comprising the step of introducing into the host cell nucleic acid molecules encoding one or more enzymes for production of the glycoprotein selected from the group consisting of mannosidases, glycosyltransferases and glycosidases.

55. (canceled)

56. (canceled)

57. (previously amended) The method of claim 35, wherein the mannosidase enzyme has optimal activity at a pH between 5.1 and 8.0.

58. (currently amended) The method of claim 35, wherein the mannosidase enzyme comprises an alpha-1,2-mannosidase catalytic domain from mouse, human, *Lepidoptera*, *Aspergillus nidulans*, *Xanthomonas manihotas* or *Bacillus* sp.

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59. (previously amended) The method of claim 54, wherein at least one of the enzymes for production of the glycoprotein is localized by forming a fusion protein between a catalytic domain of the enzyme and a cellular targeting signal peptide.

60. (previously presented) The method of claim 59, wherein the fusion protein is encoded by at least one genetic construct formed by the in-frame ligation of a DNA fragment encoding a cellular targeting signal peptide with a DNA fragment encoding a glycosylation enzyme or catalytically active fragment thereof.

61. (currently amended) The method of claim 59, wherein the catalytic domain encodes a glycosidase or glycosyltransferase selected from the group consisting of GnT I, GnT II, GnT III, GnT IV, GnT V, GnT VI, GalT, Fucosyltransferase and ST, and wherein the catalytic domain has optimal activity at a pH between 5.1 and 8.0.

62. (previously presented) The method of claim 54, wherein the nucleic acid molecule encodes one or more enzymes selected from the group consisting of UDP-GlcNAc transferase, UDP-galactosyltransferase, GDP-fucosyltransferase, CMP-sialyltransferase, UDP-GlcNAc transporter, UDP-galactose transporter, GDP-fucose transporter, CMP-sialic acid transporter, and nucleotide diphosphatases.

63. (previously presented) The method of claim 54, wherein the host expresses GnTI and a UDP-GlcNAc transporter.

64. (previously presented) The method of claim 54, wherein the host expresses a UDP- or GDP-specific diphosphatase.

65. (previously presented) The method of claim 40, wherein the one or more additional enzymes is targeted to the endoplasmic reticulum, the early, medial or late Golgi, or the trans Golgi network of the host cell.

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66. (previously presented) The method of claim 65, wherein the one or more additional enzymes is targeted by means of a cellular targeting signal peptide not normally associated with the enzyme.

67. (previously presented) The method of claim 40, wherein the one or more additional enzymes is selected to have a pH optimum within 1.4 pH units of the average pH optimum of glycosylation-related enzymes where the enzyme is localized.

68. (previously presented) The method of claim 54, wherein at least one nucleic acid molecule encoding one or more enzymes is introduced into the host cell by integration into the host cell chromosome.

69. (previously presented) The method of any one of claims 40, 54 or 68, wherein at least one of the encoded enzymes is GnTI.

70. (currently amended) A method for producing a recombinant glycoprotein comprising an N-glycan structure that comprises a GlcNAcMan₅GlcNAc₂ glycoform attached to a recombinant protein in a lower eukaryotic host cell, the cell engineered to produce N-glycan structures having an excess of 30 mole % of a Man₅GlcNAc₂ glycoform that can serve as a substrate for GlcNAc transferase I *in vivo*, the method comprising the step of expressing in said host cell an enzyme comprising:

(a) a catalytic domain having GlcNAc transferase I activity selected to have a pH optimum within 1.4 pH units of the average pH optimum of glycosylation-related enzymes in the subcellular location where the domain is targeted; and

(b) a cellular targeting signal peptide not normally associated with the catalytic domain selected to target the catalytic domain to the ER or Golgi apparatus of the host cell.

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71. (currently amended) A method for producing a recombinant glycoprotein comprising an N-glycan structure that comprises a GlcNAcMan₅GlcNAc₂ glycoform in a lower eukaryotic host cell that does not display alpha-1,6 mannosyltransferase activity with respect to the N-glycan on a glycoprotein, the method comprising the step or steps of introducing into the host cell at least two enzymes comprising:

(a) a catalytic domain having alpha-1,2 mannosidase activity selected to have a pH optimum within 1.4 pH units of the average pH optimum of glycosylation-related enzymes in the subcellular location where the domain is targeted; and

(b) a cellular targeting signal peptide not normally associated with the catalytic domain selected to target the catalytic domain to the ER or Golgi apparatus of the host cell;

the second enzyme comprising:

(a) a catalytic domain having GlcNAc transferase I activity selected to have a pH optimum within 1.4 pH units of the average pH optimum of glycosylation-related enzymes in the subcellular location where the domain is targeted; and

(b) a cellular targeting signal peptide not normally associated with the catalytic domain selected to target the catalytic domain to the ER or Golgi apparatus of the host cell.

72. (currently amended) The method of any one of claims 70, 71, or 74-77, further comprising the step of introducing into the host cell a nucleic acid encoding a UDP-GlcNAc transporter.

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73. (previously presented) The method of any one of claims 35, 40, 54, 70-72 or 74-77, further comprising the step of analyzing a glycosylated protein or isolated *N*-glycan produced in the host cell by one or more methods selected from the group consisting of: (a) mass spectroscopy such as MALDI-TOF-MS; (b) liquid chromatography; (c) characterizing cells using a fluorescence activated cell sorter, spectrophotometer, fluorimeter, or scintillation counter; (d) exposing host cells to a lectin or antibody having a specific affinity for a desired oligosaccharide moiety; and (e) exposing cells to a cytotoxic or radioactive molecule selected from the group consisting of sugars, antibodies and lectins.

74. (new) A method for producing a recombinant glycoprotein comprising an *N*-glycan having a GlcNAcMan₅GlcNAc₂ glycoform in a lower eukaryotic host cell that displays alpha-1,2, mannosidase activity and that does not display an alpha-1,6 mannosyltransferase activity with respect to the *N*-glycan on a glycoprotein, the method comprising the step of introducing into the ER or Golgi apparatus of the the host cell an enzyme comprising:

(a) a catalytic domain having GlcNAc transferase I activity selected to have a pH optimum within 1.4 pH units of the average pH optimum of glycosylation-related enzymes in the subcellular location where the domain is targeted; and

(b) a cellular targeting signal peptide not normally associated with the catalytic domain selected to target the catalytic domain to the ER or Golgi apparatus of the host cell;

whereby, upon expression in the host cell, a recombinant glycoprotein having a GlcNAcMan₅GlcNAc₂ glycoform is produced.

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75. (new) A method for producing a recombinant glycoprotein comprising an N-glycan having a GlcNAcMan₃GlcNAc₂ glycoform in a lower eukaryotic host cell that displays alpha-1,2, mannosidase activity and GlcNAc transferase I activity and that does not display an alpha-1,6 mannosyltransferase activity with respect to the N-glycan on a glycoprotein, the method comprising the step of introducing into the ER or Golgi apparatus of the the host cell an enzyme comprising:

(a) a catalytic domain having mannosidase II activity selected to have a pH optimum within 1.4 pH units of the average pH optimum of glycosylation-related enzymes in the subcellular location where the domain is targeted; and

(b) a cellular targeting signal peptide not normally associated with the catalytic domain selected to target the catalytic domain to the ER or Golgi apparatus of the host cell;

whereby, upon expression in the host cell, a recombinant glycoprotein having a GlcNAcMan₃GlcNAc₂ glycoform is produced.

76. (new) A method for producing a recombinant glycoprotein comprising an N-glycan having a GlcNAc₂Man₃GlcNAc₂ glycoform in a lower eukaryotic host cell that displays alpha-1,2, mannosidase activity, a GlcNAc transferase I activity and a mannosidase II activity, and that does not display an alpha-1,6 mannosyltransferase activity with respect to the N-glycan on a glycoprotein, the method comprising the step of introducing into the ER or Golgi apparatus of the the host cell an enzyme comprising:

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(a) a catalytic domain having GlcNAc transferase II activity selected to have a pH optimum within 1.4 pH units of the average pH optimum of glycosylation-related enzymes in the subcellular location where the domain is targeted; and

(b) a cellular targeting signal peptide not normally associated with the catalytic domain selected to target the catalytic domain to the ER or Golgi apparatus of the host cell;

whereby, upon expression in the host cell, a recombinant glycoprotein having a GlcNAc₂Man₃GlcNAc₂ glycoform is produced.

77. (new) The method of any one of claims 74-76, wherein one or more of the alpha-1,2 mannosidase, GlcNAc transferase I, GlcNAc transferase II and mannosidase II activities is expressed from an exogenous DNA introduced into the lower eukaryotic host cell.

78. (new) A lower eukaryotic host cell produced by any one of the methods of claims 35-77.